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Langmuir silk films

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LANGMUIR BLODGETT FILMS
BOMBYX MORI MEMBRANES

THIN FILMS
SILK

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Formation and Characterization of Langmuir Silk Films

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Langmuir films were formed from regenerated (solubilized cocoons) *Bombyx mori* silk. Pressure/area isotherms and deposition studies indicate the formation of stable films; however, due to the molecular weight and solubility nature of silk it has been difficult to determine the area occupied by each protein polymer chain. The thin films were successfully transferred on to various solid supports for analysis. Ellipsometry data indicate a film thickness of 11.6 to 11.9 Å. Infrared spectrometry (ATR) shows wavenumbers at the amide bands of 1624 (amide I), 1522 (amide II), 1258 (amide III), and 700.8 (amide V), indicative of a silk II structure. TEM micrographs show pores of 300–3500 Å in diameter and electron diffraction analysis indicates a polycrystalline structure.

Introduction

Bombyx mori silk consists of two major types of proteins, fibroin and sericin. Fibroin is the protein that forms the filaments of silkworm silk and gives silk its unique physical, chemical, and biological properties. Sericins are a group of gummy proteins which bind the fibroin filaments. Unique properties of fibroin include its ability to form antiparallel β -sheet secondary structures, assemble into cast films for immobilization matrices,^{1–5} form fibers which are stable to most solvents including water, exhibit biological compatibility, and possess good tensile strength and elasticity properties in fiber form.^{6,7} As a result of these properties, silk has been used in various applications including fibers for textiles and surgical thread⁸ and as a matrix material for biosensors to physically entrap reactive biological materials.^{3–5,9} Fibroin undergoes a phase transition during processing in the silkworm, from a water-soluble protein in the silk-producing gland (silk I) to a water-insoluble protein fiber (silk II).^{10,11} Our interests lie in the membrane properties of silk and the utilization of the phase transition (silk I to silk II) in the processing of water-soluble fibroin polymer to water-insoluble films.

B. mori silk fibroin has been used as an immobilization matrix for enzymes such as glucose oxidase,^{1–4} alkaline phosphatase,¹² peroxidase,⁵ and invertase.¹³ An advantage of the silk membrane as an immobilization matrix is its ability to entrap enzymes without the need to covalently bind the enzymes to the matrix. The entrapment process is accomplished by physical, chemical or mechanical treatment of the membrane (e.g., temperature, pH, solvent,

mechanical shear, or stretch) which induces the phase transition to silk II.

Silk structures studied for phase transitions as immobilization matrices have been in the form of cast membranes. The cast silk membranes have good properties as membrane materials. However, the casting process has limitations. There is limited control over the thickness of the membrane or the density and the orientation to the polymer chains. Since the desired properties of these membranes, including selective permeability, entrapped enzyme activity, and mechanical integrity, are dependent on conformation, density, and orientation of the polymer chains, new processing techniques to control these properties would be extremely valuable. The Langmuir-Blodgett (LB) technique is used in this study in an attempt to enhance the control over the physical processing of silk protein. The long term goals for these films include tailorable permeability and enhanced mechanical properties through improved control over molecular architecture.

The dispersion of water-soluble polymers at an air-water interface, including most proteins, is a complicated process requiring a reduction of the free energy of the system.¹⁴ At low surface pressure the completely spread monolayer is the thermodynamically most favorable state. It is difficult to achieve equilibrium of large macromolecules which contain many chain segments at the air-water interface.¹⁴ Ordered regions of proteins, such as those joined by disulfide bridges or containing α helices, are often retained at the surface of an air-water interface.¹⁵ With this difficulty realized, recent interest in Langmuir-Blodgett films with proteins has been primarily with mixed monolayer systems in which the protein is added to a lipid monolayer.^{16–19} These mixed monolayer systems have potential as biosensors and serve as mimics for membrane bilayers.^{20–22}

We describe here the formation and characterization of natural silk fibroin films using the Langmuir-Blodgett

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technique. Basic information regarding the physical characteristics of the thin films is obtained from pressure-area isotherms, electron micrographs, and ellipsometry. Analysis of the silk fibroin LB films with infrared spectroscopy and electron diffraction provides insight into the silk conformation favored at ambient temperature and the expected polycrystalline order of the silk film.

Experimental Section

Preparation of Soluble Silk. *B. mori* cocoons with the chrysalides (stage of development between larva and moth) removed were cut into approximately 5 mm × 5 mm pieces. The cocoon material was boiled in distilled water for 2 h with changes of water to solubilize and remove the sericin. The remaining matted silk (primarily fibroin) was dried at 40 °C overnight. A 5% aqueous solution of the dried silk in 9.3 M LiBr was mixed in a rotary shaker (200 rpm) at 40 °C. The solubilized silk was then dialyzed (Spectrapor, 12,000–14,000 MW cut off dialysis tubing, Spectrum Medical Industries, Inc., Los Angeles, CA) for 3 days against one change of distilled water. The final working concentration after dialysis was approximately 2.5% silk.

Pressure/Area Isotherms. A Lauda Filmbalance FW2 with an IBM Computer Personal System/2 Model 30 286 and Hewlett-Packard 7475A plotter was used for the pressure/area isotherms. To create silk films a 2.5-mL Pasteur pipet was used to apply the solubilized silk to the surface of the trough. A Pasteur pipet was used in lieu of a syringe to minimize crystallization (shear induced) of the silk during application. The soluble silk was applied to a Milli-Q water subphase at a temperature of 24 °C.

Infrared Spectroscopy. FTIR attenuated total reflectance (ATR) analysis of the soluble silk was performed on a Nicolet 20SXB infrared spectrometer, Nicolet 1180 computer, and Zeta 8 plotter (Madison, WI) with an accessory holder for ATR (Harrick Scientific Co., Ossining, NY). The germanium prisms used to collect the silk fibroin LB film were Harrick Model EJ3122, size 50 × 10 × 2 mm, spp 45° (Harrick Scientific Co.). The silk films were vertically deposited onto the prisms from a Lauda MGW Filmbalance at a pressure of 16.7 mN/m, a dipping speed of 0.2 cm/min, and a temperature of 20 °C.

Transmission Electron Microscopy. Transmission electron micrographs and electron diffraction patterns of the soluble silk were performed on a Hitachi H 600 (Rockville, MD). The silk film was collected on nickel T1000 grids (Ernest F. Fullam, Inc., Schenectady, NY) at 24 °C and a compression pressure of 16.7 mN/m. The grids were hand dipped using tweezers.

Ellipsometry. A thin film ellipsometer, type 43603-200E (Rudolph Research, Flanders, NJ), was used to characterize silk films deposited on frosted glass slides (76 × 25 × 1 mm) under the same conditions used for FTIR samples. Ellipsometry analysis was at 6328 Å. The refractive index of silk fiber, $n_1 = 1.591$ and $n_2 = 1.538$, was used as a guide in the analysis of the samples.²³

Results and Discussion

Figure 1 is a pressure/area isotherm of the soluble silk. The steep slope of the curve indicates film formation. It should be noted that the x axis of the isotherm is in arbitrary units because the silk fibroin has an estimated molecular weight of 350–415 kDa which exceeds the limits of the Lauda software program. A molecular weight of 75.53 Da was used as the basis of the calculation, derived from the amino acid composition of fibroin^{24,25} and is based on the average weight of each amino acid monomer in the silk fibroin polymer. It has been difficult to obtain an accurate quantitation of the area per molecule due to the complex secondary structure of the silk fibroin protein.

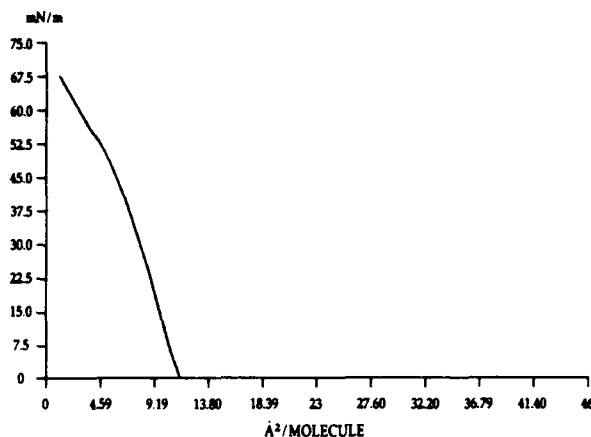


Figure 1. Typical pressure/area isotherm of solubilized silk fibroin at 24 °C.

The molecular structure of silk fibroin does not exhibit the typical amphipathic character of LB materials suitable for monolayer formation. Also, silk fibroin exhibits unique solubility characteristics which can make the material difficult to work with in a LB system. Silk fibroin is insoluble in volatile nonpolar solvents often used in the application of surfactants to an aqueous subphase. Silk fibroin is also insoluble in dilute acids and alkali and resistant to most proteolytic enzymes^{8,10} but soluble in 9.3 M LiBr aqueous solution. After dialysis, the solubilized silk fibroin remains in solution if undisturbed. When the silk is applied to the trough surface, a portion of the polymer enters the aqueous subphase. This event is evident from the protein residue observed on the bottom of the trough upon cleaning. This unusual solubility behavior adds to the difficulty in obtaining an accurate determination of the area occupied per polymer chain. However, it should be noted that the pressure-area isotherms obtained are fairly reproducible, within ± 3 Å²/molecule for each run. These results suggest that the problems encountered with using silk as a LB forming material behave consistently enough to encourage further study.

The silk fibroin films demonstrate excellent stability and transfer properties indicative of a well-behaved Langmuir–Blodgett system. Constant pressure was maintained after a 30–40-min stabilization period at various pressures (10, 15, 20, 25, 30, 35 mN/m) with no indication of film collapse. Films remained stable overnight (16 h) without a change in area when annealed at our usual transfer pressure of 16.7 mN/m. In Figure 1, a typical isotherm for silk, there is no sudden drop in surface pressure with compression, suggestive of a collapsed film. Upon complete collapse of the monolayer, it was possible to remove very long, elastic-like fibers at the end of a pipet. The steady rise in surface pressure of the isotherm and unusual physical properties of the collapsed film may be indicative of the characteristic strength and elasticity properties observed in bulk silk systems.^{6,7}

Figure 2 is a hysteresis study of the silk LB film which provided further evidence of the stability of the system. In Figure 2 there are four compression and expansion curves with a 1-min pause between each cycle and a maximum designated pressure of 25 mN/m. There is little or no difference in the height, slope, or area of the four curves, indicative of a stable system. Despite the small hysteresis effect, there is a drop in pressure during the 1-min pause on each cycle after attaining the designated maximum pressure (25 mN/m). As stated previously, once the fibroin film is stabilized it maintains the same area

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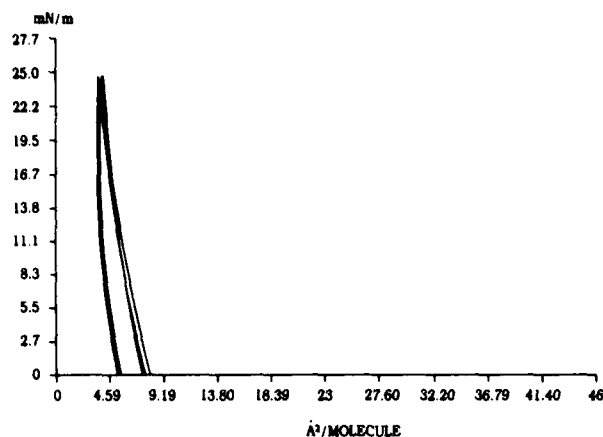


Figure 2. Hysteresis study of a solubilized silk fibroin thin film showing four curves run at a maximum pressure of 25 mN/m with a 1-min pause at the maximum pressure.

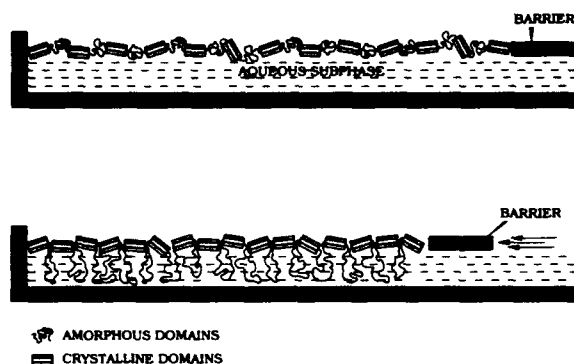


Figure 3. Model depicting interactions at the air-water interface during thin film formation.

Table I. Ellipsometry Data on Silk Fibroin Thin Film Deposited on Frosted Glass Slides

film characteristic	3 layers	5 layers	7 layers
multilayer thickness, Å	37	65	94
calculated monolayer thickness, Å	12.3	13	13.4

and pressure for hours; thus the drop in pressure in this hysteresis study indicates the film did not yet have sufficient time to stabilize. However, it appears that after each cycle the film is able to recover and exhibit a near identical curve on the next run. This again may be an indication of the unusual mechanical properties characteristic to silks.

Figure 3 is a model of what may be occurring during the equilibration phase for the deposition of stable silk films. Once the designated pressure is achieved, the fibroin, composed of amorphous and crystalline regions, is positioned at the air-water interface. During equilibration the more flexible amorphous domains may be squeezed to some degree into the subphase and the barrier moves in to compensate for the loss of polymer at the surface while maintaining constant pressure. Subsequently, this leaves primarily the crystalline domains at the interface to form a stable film. This behavior has permitted us to deposit the silk films on to various substrates in order to employ a number of analytical tools for further characterization.

Table I presents the ellipsometry data for the deposited silk films. Many biomolecular materials exhibit Y- or Z-type deposition.¹⁵ We believe that Y-type deposition is characteristic of the silk fibroin based on our observations of the change in area and shape of the meniscus at the air/water interface during each vertical pass. The average thickness of each layer determined from the data in Table

I was approximately 11.6–11.9 Å. The ellipsometry data indicated that the average thickness increased with the number of layers deposited. Therefore, by extrapolation back to a single layer, a value of 11.6–11.9 Å was established.

Silk from *B. mori* consists of antiparallel β sheets as first described by Marsh et al.²⁶ The fibroin consists of both crystalline (short side chain amino acid monomers glycine, alanine, serine) and amorphous (amino acids with bulkier side chains) domains. There have been two types of crystalline structures proposed for silk, silk I and silk II. For silk II, the insoluble and more stable form of silk, the reported unit cell based on X-ray diffraction data is composed of three chains having an interchain distance of 9.4 Å, a fiber axis distance of 6.97 Å, and an intersheet distance of 9.2 Å.²⁶ A single chain of the silk crystalline domain is 4.5–4.7 Å thick while the amorphous chains vary in their thickness. The model suggested in Figure 3, where the crystalline domains are at the air/water interface and the amorphous domains are buckled into the subphase, is consistent with the ellipsometry value of 11.6–11.9 Å. The amorphous domains would not be uniform in their arrangement underneath the crystalline domains creating an irregular surface with the ellipsometry value representing the average thickness of the silk film deposited on the substrate.

Silk I is also of interest due to its metastable nature, its role in the natural processing of silk, and its unresolved structure. Silk I is the conformation of the soluble form of silk fibroin that rapidly undergoes a phase transition to the insoluble silk II conformation.^{10,11} This transition can be activated by mechanical agitation, exposure to hydrophilic organic solvents, or temperature changes.^{27–31} Due to this instability, experimental studies with silk I have been primarily on samples of low orientation (attempts to improve orientation result in conversion to silk II). Therefore, elucidation of the structure of silk I has depended on molecular modeling and comparisons with the limited available experimental evidence.¹⁰ A number of models have been proposed, including the Lotz and Keith³² crankshaft model based on poly(L-Ala-Gly) lamellar crystals (intersheet distance of 14.4 Å) and a recent model of Fossey et al.¹¹ based on conformational energy calculations with copolymers of Gly-Ala and stacked sheets (intersheet distance of 11.3 Å). Both of these intersheet distances are in close agreement to our estimated film thickness.

Infrared spectroscopy was used to partially characterize the structure of silk in the LB film. Yoshimizu and Asakura,³³ in a study on cast films with a thickness of 100–250 μ m, employed FTIR (ATR) to determine the conformational transition of the silk membrane surface treated with methanol. The absorption bands observed for membranes treated with methanol had frequencies of 1625 (amide I), 1528 (amide II), and 1260 cm^{-1} (amide III), characteristic of a silk II structure. Membranes without methanol treatment showed absorption bands at 1650 (amide I), 1535 (amide II), and 1235 cm^{-1} (amide II) which

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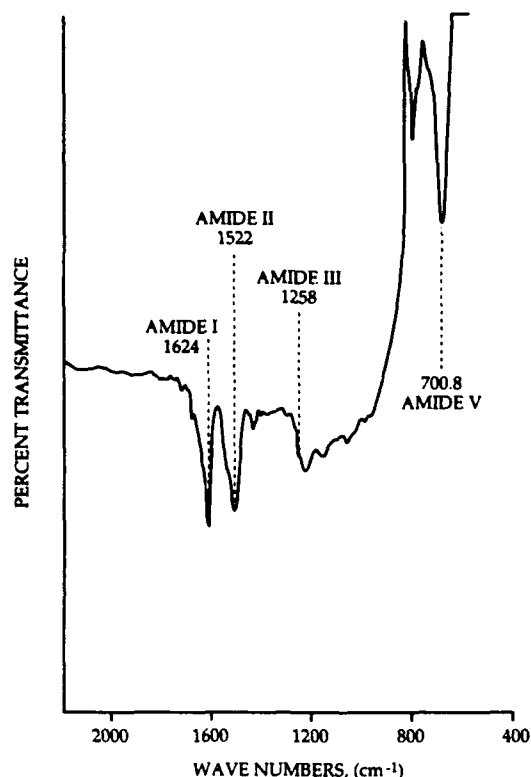


Figure 4. Transmission IR spectra of 11 layers of silk fibroin collected on a germanium prism at 20 °C and a pressure of 16.7 mN/m.

were assigned the random coil conformation. Silk I and random coil conformations cannot be distinguished by IR.³⁴

FTIR (ATR) results with a regenerated silk fibroin LB film are shown in Figure 4. A total of 11 silk layers were deposited on a germanium prism. Absorption bands were observed at 1624 (amide I), 1522 (amide II), and 1258 cm⁻¹ (amide III), which compares favorably with the silk II spectrum reported by Yoshimizu and Asakura.³³ The pronounced shoulder observed at 1258 cm⁻¹ for the LB silk fibroin film is a critical feature to distinguish silk II from random coil/silk I in cast silk films treated with methanol. In addition, Asakura et al.³⁴ observed that the amide V band had a frequency of 700 cm⁻¹ for a silk II compared to a 650 cm⁻¹ for the random coil conformation. In Figure 4 the amide V absorption band for the LB silk film was 700.8 cm⁻¹.

There are at least two possible explanations for the dominance of a silk II structure in LB silk films. In the absence of an externally applied physical, chemical, or mechanical treatment of the LB silk film, the drying process itself may contribute to the silk II character. Magoshi³⁵ and Magoshi et al.²⁹ studied spherulite formation in silk fibroin and found that drying temperature and rate are critical in the determination of silk I or silk II. Bhat and Ahirrae³⁶ reported that micrographs of freshly formed silk films, regenerated with lithium thiocyanate solution, have spherulitic structure but after 15 days show parallel striations attributed to alignment of microfibrils. Infrared spectra of the 15 day old films indicate the appearance of bands at 1560 cm⁻¹, not seen with the freshly poured film. They suggest a partial crystallization of the film and a transformation to a silk II conformation.

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Figure 5. A transmission electron micrograph of the deposited silk fibroin thin film at 28000X.

Another explanation for the dominance of the silk II structure in the LB films may be mechanical forces present during the application, compression, and/or transfer/deposition of the solubilized silk fibroin on the surface of the trough. During application with the Pasteur pipet some shear may induce, in part, a silk II conformation. The silk II conformation, due to its insolubility in the aqueous subphase, would remain to form the thin film. Another possible source of mechanical shear is the stretching of silk fibroin during transfer and deposition on to solid supports. Once a silk fibroin film is formed, there may be resistance to transfer and deposition by interchain forces between the polymer chains. The energy needed to break these interchain forces may contribute to the phase transition of the deposited material.

Figure 5 is a TEM of the silk fibroin LB film. The larger dark areas in the micrograph are presumed to be undissolved silk material while the small dark areas are crystals of LiBr not removed during dialysis. The clear or spherical areas in the micrograph are pores in the film. These pores appear irregularly throughout the film and may arise during film drying on the TEM grid. This is supported by the absence of these holes in some films. The pores range in size from 300 to 3500 Å in diameter. The physical appearance of the film is affected by the drying and processing conditions. Altering the temperature and pH of the subphase imparts changes in the physical appearance of the silk fibroin films in TEM micrographs (unpublished data). Further studies are underway to correlate the physical environment of the subphase with the structure of the LB silk film formed.

Figure 6 shows a micrograph of an electron diffraction pattern of an LB silk fibroin film from Figure 5. The electron diffraction pattern is typical of a polycrystalline material which is characteristic of silk. Minoura et al.³⁰ and Magoshi et al.³² have published X-ray diffraction data on cast silk thick films which have similar patterns as those observed here for the LB silk fibroin film. Asakura et al.³⁴ and Minoura et al.,³⁰ using X-ray diffraction,

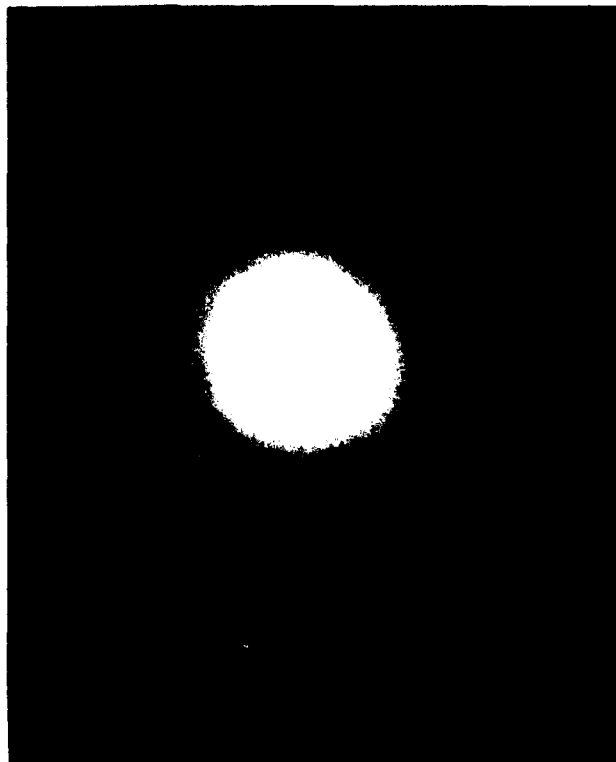


Figure 6. Electron diffraction pattern of silk fibroin thin film.

observed that the preparation and physical treatment of silk films are critical factors in determining whether silk I or silk II structures form. We have yet to confirm with electron diffraction whether the silk film present in this micrograph is silk I or silk II. Further work is underway to utilize electron diffraction to determine the structure of silk in the films.

Based on the IR data discussed earlier we expect a silk II conformation to be present for the conditions under which these films were prepared. To determine the conformation characteristic of the LB silk film individual reflections within the electron diffraction pattern would be critical for structure identification. Reflections with d spacings of 9.70, 4.69, and 4.30 Å would indicate a silk II conformation while reflections at 7.25 and 4.50 Å would be indicative of a silk I conformation. Under the appropriate conditions, using the Langmuir method it may be possible to obtain a well-oriented silk I film. This would provide a unique opportunity to experimentally characterize the silk I structure since this structure has eluded definitive structural characterization due to its metastable state.

Conclusions

Regenerated *Bombyx mori* silk can be used to form LB films. Although the area occupied by the protein chains cannot be determined from the pressure/area isotherms, the LB films are stable and were successfully transferred. The ellipsometry data provided an estimated film thickness of 11.6–11.9 Å. The infrared spectra of the LB silk films agree with data from cast silk films and indicate a silk II structure. Polycrystalline order was observed in the thin films by electron diffraction analysis. The Langmuir–Blodgett approach to thin silk fibroin films may prove useful in gaining insight into structural changes in silk and clarifying the silk I structure.

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